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Increased apoptosis of parasympathetic but not enteric neurons in mice lacking GFRα2

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Abstract

Enteric neurons, unlike sympathetic and sensory neurons that require target-derived neurotrophins for survival, do not undergo classical caspase-3-mediated programmed cell death (PCD) during normal development. Whether parasympathetic neurons in the pancreas, which originate from a subpopulation of enteric nervous system (ENS) precursors, or other parasympathetic neurons undergo PCD during normal mammalian development is unknown. In GFRα2-deficient mice, many submandibular and intrapancreatic parasympathetic neurons are missing but whether this is due to increased neuronal death is unclear. Here we show that activated caspase-3 and PGP9.5 doubly positive neurons are present in wild-type mouse pancreas between embryonic day E15 and birth. Thus, in contrast to ENS neurons, intrapancreatic neurons undergo PCD via apoptosis during normal development. We also show that, in GFRα2-deficient mice, most intrapancreatic neurons are lost during this late fetal period, which coincides with a period of increased apoptosis of the neurons. Since the percentage of BrdU and Phox2b doubly positive cells in the fetal pancreas and the number of intrapancreatic neurons at E15 were similar between the genotypes, impaired precursor proliferation and migration are unlikely to contribute to the loss of intrapancreatic neurons in GFRα2-KO mice. Caspase-3-positive neurons were also found in GFRα2-deficient submandibular ganglia around birth, suggesting that parasympathetic neurons depend on limited supply of (presumably target-derived neurturin) signaling via GFRα2 for survival.

Keywords: Activated caspase-3; GDNF; Intrapancreatic neurons; Neurturin; Parasympathetic nervous system; Programmed cell death; Submandibular ganglion; TUNEL

Introduction

In developing sympathetic and sensory ganglia, the number of neurons is matched to the size of the target tissue by target-derived neurotrophins required for the survival of these neurons (Huang and Reichardt, 2001; Glebova and Ginty, 2005). In contrast, caspase-3- and BAX-mediated programmed cell death (PCD) is not observed during normal development of the enteric nervous system (ENS), and the number of ENS neurons is thought to be regulated by the proliferation of neuronal precursors (Gianino et al., 2003). Little is known about how the number of parasympathetic neurons is adjusted during development in mammals. In the chick ciliary ganglion, about half of the neurons undergo PCD during development (Landmesser and Pilar, 1974), and target-derived neurturin and ciliary neurotrophic factor CNTF are candidate survival factors for chick ciliary neurons *in vivo* (Finn et al., 1998; Hashino et al., 2001). On the other hand, parasympathetic neurons in frog cardiac ganglia match the growing target size with increased precursor proliferation (Heathcote and Sargent, 1987a), and PCD is not considered to play a role in their development (Heathcote and Sargent, 1987b). The lack of PCD in enteric and some parasympathetic neuron development may reflect the proximity of these neurons to their targets.

Mammalian parasympathetic neurons require target-derived neurturin signaling through a complex of GDNF-family receptor α2 (GFRα2) and Ret receptor tyrosine kinase (for a review, see Baloh et al., 2000; Airaksinen and Saarma, 2002). Studies with neurturin- and GFRα2-KO (knock-out) mice have shown that this signaling is important for the development of parasympathetic target innervation. GFRα2 and Ret are found

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together in many parasympathetic and enteric neurons in late fetal development, as well as postnatally, while neurturin mRNA is expressed in the targets they innervate (Widenfalk et al., 1997; Golden et al., 1999; Rossi et al., 2000; Wanigasekara et al., 2004). Cholinergic innervation of the small intestine, as well as parasympathetic innervation of the exocrine and endocrine pancreas, heart, mucosa of reproductive organs and several cranial targets such as the salivary and lacrimal glands are reduced in these mice (Enomoto et al., 2000; Heuckeroth et al., 1999; Hiltunen et al., 2000; Rossi et al., 1999, 2000, 2003, 2005; Wanigasekara et al., 2004). In addition, lack of neurturin/GFRα2 signaling leads to reduced soma size and/or reduced neuron numbers in at least some parasympathetic ganglia (Heuckeroth et al., 1999; Enomoto et al., 2000; Rossi et al., 2000). In particular, GFRα2-KO mice show a ~40% loss of submandibular neurons and a ~85% loss of intrapancreatic neurons, but the mechanism underlying the neuronal losses remains unclear (Rossi et al., 2003).

Like enteric neurons, intrapancreatic neurons form a plexus of small ganglia and develop from a subgroup of vagal neural crest-derived ENS precursors (Kirchgessner et al., 1992; Jiang et al., 2003). These precursors start to migrate from the primitive bowel to the developing pancreas presumably between embryonic day E10 and E12.5 in mice (Jiang et al., 2003; Rossi et al., 2003). The aim of this study was to determine whether intrapancreatic and submandibular parasympathetic neurons undergo PCD during normal mouse development and whether signaling via GFRα2 is required for their survival (or proliferation).

Materials and methods

Animals and tissue processing

Most experiments used wild-type and GFRα2-KO littermates from heterozygous parents maintained in a C57BL/6JOlaHsd (B6) background. At embryonic day E17.5, three additional wild-type mice in F1 hybrid (B6×129S2) background were used for pancreatic counts. Newborn (P0) wild-type and GFRα2-KO mice were from separate litters. Vaginal plug date was considered to be E0.5. Fetuses were harvested around midday, and their developmental age was confirmed afterwards (Kaufman and Bard, 1999). The mice were genotyped as described previously (Rossi et al., 1999). Newborn mice were deeply anesthetized and perfused with phosphate buffered saline (PBS) following 4% PFA in PBS (pH 7.5). Trunks and heads of fetuses and pups were fixed in 4% PFA overnight at 4 °C and then gradually dehydrated and paraffin wax-embedded. Additional fetuses were embedded in sucrose for cryosections. All animal experiments were approved by the Animal Ethics Committee of the University of Helsinki.

Immunohistochemistry

For activated caspase-3 and PGP9.5 double-label immunohistochemistry, paraffin sections were rehydrated and the endogenous peroxidase activity was blocked for 15 min in 0.5% H_2O_2 in 70% methanol. Antigen retrieval was carried out in 10 mM sodium citrate buffer, pH 6.0. The buffer was pre-heated to 70 °C, and the slides were then heated in a microwave (650 W) three times for 4 min, avoiding boiling. Slides were blocked for 1 h in 5% normal donkey serum followed by incubation in rabbit anti-activated caspase-3 (1:5000, Cell Signaling Technology, Danvers, MA) and positive cells were detected by a Cy3 Tyramide Signal Amplification (TSA) kit (PerkinElmer, Wellesley, MA). Sections were then incubated with sheep anti-PGP9.5 (1:50, Serotec, Oxford, UK) followed by a 4-h incubation in donkey anti-sheep Cy2 (1:150) for detection. In some experiments, double labeling was performed using rabbit anti-PGP9.5 (1:400, Chemicon/Millipore, Billerica, MA) and donkey anti-rabbit Cy2 (1:200) which gave similar results (data not shown). The rabbit anti-activated caspase-3 antibody (diluted 1:5000) was not recognized by the donkey anti-rabbit Cy2, confirmed when the rabbit anti-PGP9.5 was omitted from the protocol. Finally, the nuclei were stained with Hoechst 33258 (2 μg/ml in PBS, 2 min). In double-label immunohistochemistry for insulin and PGP9.5, guinea pig anti-insulin (1:3000, Abcam, Cambridge, UK) was detected using the Cy3-TSA system and followed by staining for PGP9.5 as above. All secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Slides were always incubated in primary antibodies for 40 h at 4 °C, and 0.3% Triton X-100 in PBS was used as a wash buffer.

To label proliferating cells, pregnant mice were injected intraperitoneally with BrdU (Sigma, 6 mg/100 g diluted in saline) 2.5 h before sacrifice. After blocking endogenous peroxidase as above and antigen retrieval overnight in PBS at 70 °C, 10-μm-thick cryosections were incubated 2.5 h in rabbit anti-Phox2b (1:3000, a kind gift from Dr. Pattyn (Pattyn et al., 1999)) and detected with a TSA-fluorescein system (PerkinElmer). This was followed by reblocking the peroxidase activity as above and blocking unspecific binding in 10% normal goat serum. Sections were then incubated with mouse anti-BrdU antibody (Amersham, prediluted containing nuclease and 3% normal goat serum) for 1.5 h. The BrdU antibody was detected with peroxidase-conjugated goat anti-mouse antibody (DAKO) and the TSA-Cy3 system as above.

For Phox2b-PGP9.5 double labeling, cryosections were first stained for Phox2b using the TSA-Cy3 for detection followed by staining with sheep anti-PGP9.5 as above. For terminal deoxytransferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) and PGP9.5 double labeling, paraffin or cryosections were labeled using rabbit anti-PGP9.5 as above followed by TUNEL staining according to the manufacturer's instructions (ApopTag Peroxidase In Situ Apoptosis Detection Kit) and detected using TSA-Cy3.

Nuclear area of PGP9.5-positive cell profiles

Twenty to forty intrapancreatic and submandibular PGP9.5-positive cell profiles were randomly selected from each mouse, and the areas of nuclear profiles were determined using ImagePro program (Media Cybernetics, Silver Spring, MD). The mean nuclear area of intrapancreatic PGP9.5-positive cell profiles was similar between wild-type and GFRα2-KO mice at E15.5 (WT: 63±0.3; KO: 61±1 μm^2), E16.5 (WT: 60±2; KO, 59±2 μm^2), E17.5 (WT: 70±2; KO: 69±0.2 μm^2) and birth (WT: 71±2; KO, 68±2 μm^2; $n=3$ for each genotype and time point). Thus, the relative proportion of intrapancreatic neurons between the genotypes (Fig. 2) could be calculated directly from the raw numbers of neuronal nuclear profiles. In the submandibular ganglion, the mean nuclear area (of PGP9.5-positive profiles) was smaller in GFRα2-KO mice at birth (WT: 59±2 μm^2 versus KO: 48±1 μm^2; $P<0.05$, $n=3$ in both groups) but not at E17.5 (WT: 51±1 μm^2 versus KO: 52±1 μm^2; $P=0.5$, $n=2$ in both groups).

Cell counting

PGP9.5-positive and PGP9.5-activated caspase-3 double-positive cell profiles with a clearly visible nucleus were counted by an observer blind to the genotype. For intrapancreatic ganglia, profiles were counted from serial 7-μm-thick transversal sections cut through the pancreas using systematic random sampling of 1/10 (P0.5) or 1/5 (other time points) of the sections. If PGP9.5-positive cells were located on the border of the gut and pancreas, immunopositive cells belonging to the pancreas were determined by drawing a line between the two closest points where the gut and pancreas could clearly be discerned. For comparison, neuron profiles in left paravertebral sympathetic chain and dorsal root ganglia (DRG) were counted from every 25th section at the level of the pancreas. For ENS, five or more random sections of the gut (from duodenum to colon) were counted in each animal. The percentage of apoptotic neurons was calculated as the ratio of PGP9.5-activated caspase-3 double-positive cell profiles to all PGP9.5-positive profiles. For the submandibular ganglion, apoptosis was also estimated stereologically using the Abercrombie Method 2 (http://www.nervenet.org/papers/Aberfp.html#Method2)

(Abercrombie, 1946). Briefly, the gland was cut through serially and adjacent 7-μm and 14-μm sections were collected on slides, leaving a 14-μm gap in between every section pair. Immunopositive profiles were counted from the adjacent 7-μm and 14-μm sections. Subtraction of the sum of 7-μm section counts from the sum of 14-μm section counts results in a theoretically unbiased estimate of the cell number.

Statistical analysis

All results are expressed as mean±SEM. Statistical significance of neuron profile number and nuclear area data, as well as significance of differences in the percentages of dying neurons, was determined using the two-tailed Student's *t*-test, assuming unequal variance. $P<0.05$ was considered statistically significant.

Results

Most intrapancreatic neurons in GFRα2-KO mice are lost during late fetal development

To label intrapancreatic neurons, we used antibodies against PGP9.5, a pan-neuronal marker that has previously been used to identify neurons in the fetal (E12.5 and E15) and neonatal (P0 and P4) mouse pancreas (Jiang et al., 2003; Rossi et al., 2003). Because PGP9.5 is expressed also in the progenitors of pancreatic endocrine cells in developing rats (Yokoyama-Hayashi et al., 2002), we first established using double-label immunohistochemistry for PGP9.5 and insulin that the strongly PGP9.5-positive cell profiles in our samples were not islet cells (Figs. 1a–c). From E15.5 to E17.5, some islet cells stained for PGP9.5, but the staining was very weak compared with cells in intrapancreatic ganglia, many of which were also positive for Phox2b, a marker of autonomic neurons and their precursors (Figs. 1d–e). Some ENS precursors in the developing mouse gut express pan-neuronal markers such as PGP9.5 (Sidebotham et al., 2001; Gianino et al., 2003; Young et al., 2005). Thus, although we assume that the strongly PGP9.5-positive cells in mouse intrapancreatic ganglia after E15 are differentiated neurons, we cannot exclude the possibility that some represent neuronal precursors.

Counting of intrapancreatic PGP9.5-positive profiles from GFRα2-KO and wild-type mice at different developmental time points showed that the neuronal loss develops during the late fetal period (Fig. 2). At E15.5 and E16.5, no significant difference in the total number of intrapancreatic PGP9.5-positive profiles was seen between genotypes (E15.5 WT, 1900±100, versus KO: 1700±100; E16.5 WT: 3200±500, versus KO: 2700±400), suggesting that the migration of neuronal precursors from the bowel to the pancreas is normal in GFRα2-KO mice. In contrast, at E17.5, the number of intrapancreatic neuronal profiles was significantly (~45%) lower in GFRα2-KO than in wild-type fetuses (WT: 3600±200, versus KO: 2000±200; $P<0.0005$). By birth, GFRα2-KO mice had ~75% less intrapancreatic PGP9.5-positive profiles than wild-type mice (WT: 3400±200, versus KO: 900±100; $P<0.005$), indicating that most of the ~85% intrapancreatic neuron loss seen at postnatal day P4 (Rossi et al., 2003) develops during the last days of fetal life (Fig. 2).

Proliferation of intrapancreatic neuronal precursors is unaffected in GFRα2-deficient mice

To address whether impaired precursor proliferation would contribute to the loss of intrapancreatic neurons in GFRα2-KO mice, we counted the percentage of Phox2b-positive cells in E15.5 and E17.5 pancreatic ganglia that were also BrdU-positive (Fig. 3). The assessment of colocalization was unambiguous since both markers labeled the nucleus. The percentage of proliferating Phox2b-positive nuclei was significantly higher at E15.5 than at E17.5 (as expected), but

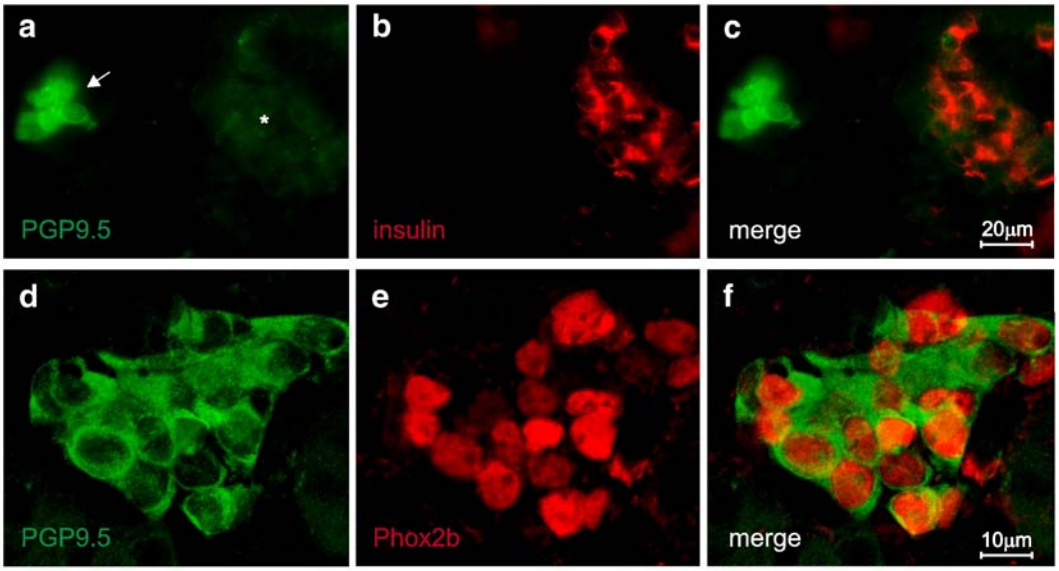

Fig. 1. (a–c) PGP9.5 (green) is strongly expressed in E17.5 wild-type intrapancreatic ganglia (arrow), whereas islet cells identified by insulin antibody (red) show only faint PGP9.5 staining (asterisk) and are thus easily distinguished from the neurons. (d–f) Confocal microscopic images of an E15.5 wild-type intrapancreatic ganglion. Cells strongly positive for PGP9.5 (green) also express transcription factor Phox2b (red).

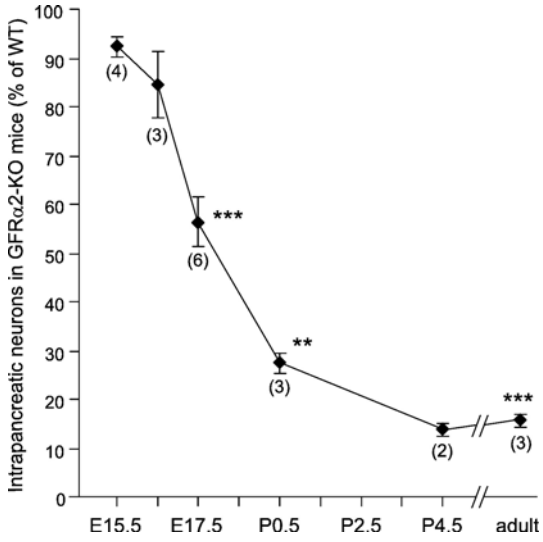

Fig. 2. Comparison of numbers of intrapancreatic PGP9.5-positive cell profiles between GFRα2-KO and wild-type mice at different developmental time points. At E15.5, no significant difference exists in the number of PGP9.5-positive profiles between genotypes ($P=0.4$). Soon thereafter, the relative number of intrapancreatic PGP9.5-positive profiles starts to decrease in GFRα2-KO fetuses, reaching statistical significance at E17.5, when the KO mice have ~45% fewer intrapancreatic neurons than wild-type animals. At birth, the deficit in KO intrapancreatic neuron profile number is already close to the final deficit (~85% loss) observed from P4 onwards. P4 and adult data are from a previous study (Rossi et al., 2003) and are shown for comparison. The number of animal pairs analyzed at each time point is indicated in parentheses. **, $P<0.005$; ***, $P<0.0005$ compared with wild-type mice.

similar between the genotypes (E15 WT 6.3±0.4%, KO 7.1±0.5%, E17.5 WT 2.1±0.4%; KO 2.7±0.5%, $n=2$ in both genotypes and time points). These results suggest that the proliferation of intrapancreatic neuronal precursors is not impaired in GFRα2-KO mice.

Developing intrapancreatic but not enteric neurons undergo PCD that is augmented in the absence of GFRα2

Consistent with a previous study reporting the absence of PCD in the developing mouse ENS (Gianino et al., 2003), the percentage of PGP9.5-expressing cells in the gut that were positive for activated caspase-3 was very low at all time points examined in both wild-type and GFRα2-KO mice (Figs. 4 and 5a) (E15.5 WT: 0.04±0.02% versus KO: 0.1±0.03; E16.5 WT: 0.11±0.02% versus KO: 0.17±0.03%; E17.5 WT: 0.05±0.01% versus KO: 0.05±0.01%; P0 WT: 0.02±0.01% versus KO: 0.04±0.02%). As a positive control, we counted caspase-3-positive neurons in paravertebral sympathetic (Fig. 5b) and spinal sensory ganglia at the pancreas level. The period of PCD in mouse sympathetic ganglia starts around E15 and continues postnatally (Francis et al., 1999), whereas most cell death in spinal sensory ganglia occurs before E15 (Farinas et al., 1996; White et al., 1998). In accord with this, the percentage of apoptotic (activated caspase-3-positive) neurons increased towards birth in sympathetic paravertebral ganglia, while the percentage was high at E15.5 and then decreased progressively towards birth in DRG (Fig. 4). The percentages of apoptotic cells in these ganglia were similar between genotypes, consistent with our previous findings that signaling via GFRα2 is not important for the survival of sympathetic or DRG neurons during development (Rossi et al., 1999; Lindfors et al., 2006). We also tried to confirm the lack of PCD in developing ENS using TUNEL/PGP9.5 double staining. Unfortunately, TUNEL-positive cells in the peripheral nervous system were often PGP9.5-negative (Supplementary Figure). Nevertheless, while relatively many (39/1058) TUNEL-positive cells were detected in E17.5 sympathetic chain ganglia, as expected, very few (0/426) TUNEL-positive cells were detected in close vicinity of PGP9.5-positive enteric neurons. This is consistent with the result obtained using activated caspase-3 (see Fig. 4) indicating that very few enteric neurons undergo apoptosis in the fetal mouse.

In contrast to the virtual lack of apoptosis in developing ENS, despite the reported common developmental origin of enteric and intrapancreatic neurons, cell profiles double-positive for PGP9.5 and activated caspase-3 were relatively numerous in wild-type mouse intrapancreatic ganglia between E15 and P0 (Figs. 4 and 5c–f). At E15.5, no significant difference existed in the percentage of dying PGP9.5-positive cell profiles in the pancreas between genotypes (WT: 1.1±0.1%, KO: 1.2±0.1%). On the contrary, apoptosis was significantly increased in the intrapancreatic ganglia from GFRα2-KO mice (as compared with wild-type littermates) at E16.5 (WT: 0.8±0.05% versus KO: 3.0±0.2%; $P=0.01$) and E17.5 (Figs. 5g–j; WT: 0.7±0.2% versus KO: 4.1±0.6%; $P<0.005$) — i.e. at the same time the loss of intrapancreatic

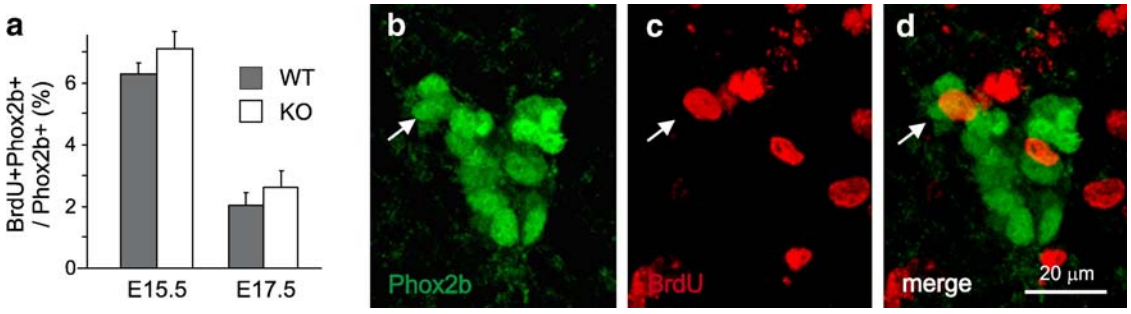

Fig. 3. Proliferation of intrapancreatic neuronal precursors in wild-type and GFRα2-KO mouse fetuses. (a) Percentage of proliferating neuronal precursors (ratio of BrdU/Phox2b double-positive profiles to Phox2b-positive profiles) in E15.5 and E17.5 pancreas is similar between the genotypes. (b–d) Confocal microscopic image of E15.5 GFRα2-KO mouse pancreas demonstrates a proliferating BrdU/Phox2b double-positive intrapancreatic precursor (arrow).

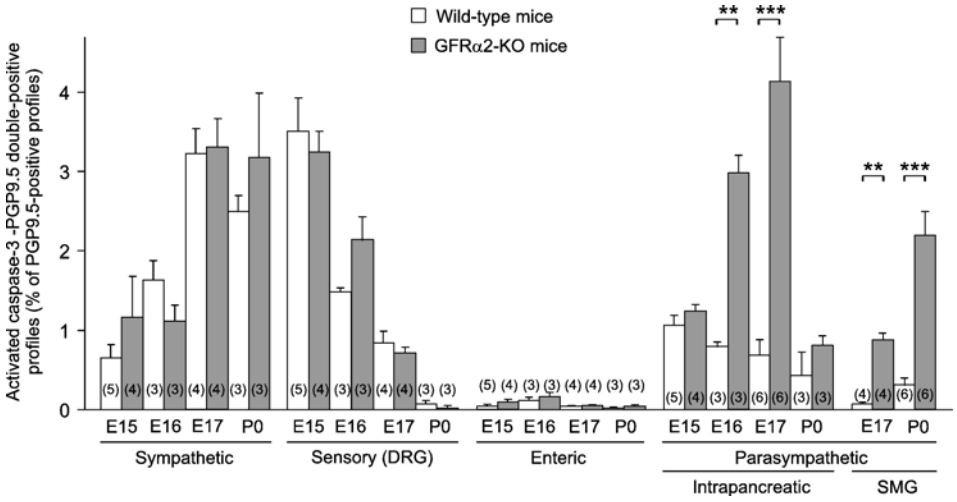

Fig. 4. Percentage of apoptotic neurons (ratio of cell profiles double-positive for activated caspase-3 and PGP9.5 to all PGP9.5-positive profiles) in different peripheral ganglia of E15.5, E16.5, E17.5 and P0.5 wild-type and GFRα2-KO mice. Percentage of activated caspase-3-positive neurons is significantly increased in GFRα2-KO mice in the intrapancreatic (at E16.5 and E17.5) and submandibular (at E17.5 and P0) ganglia. No differences between the genotypes are observed in the other ganglia. Note that, while intrapancreatic neurons undergo PCD during normal development, PCD is virtually lacking in the developing ENS. In sympathetic paravertebral ganglia, PCD increases towards birth (and continues postnatally), whereas in DRG, the period of PCD is over by birth. For SMG, the number of glands analyzed and for the other ganglia the number of animals analyzed are indicated in parentheses. **, $P=0.01$; ***, $P<0.005$ compared with wild-type mice.

neurons was shown to develop in GFRα2-KO mice. At birth, when the loss of these neurons was already close to the final level (Fig. 2), the percentage of apoptotic neurons was no longer clearly elevated in GFRα2-KO mice (WT: 0.4±0.3% versus KO: 0.8±0.2%; $P=0.29$). Thus, intrapancreatic neurons undergo PCD, and increased apoptosis contributes to the loss of intrapancreatic parasympathetic neurons in GFRα2-KO mice during the last days of fetal development.

Signaling via GFRα2 is also important for the survival of submandibular neurons

Increased neuronal cell death in GFRα2-KO intrapancreatic ganglia prompted us to examine whether defects in cell survival could also explain the previously reported ~30% loss of submandibular neurons in newborn GFRα2-KO mice (Rossi et al., 2000). Apoptosis (i.e. percentage of activated caspase-3-positive neurons) was significantly increased in GFRα2-KO submandibular ganglia at birth, estimated by standard profile counting (WT: 0.3±0.1% versus KO: 2.2±0.3%; $P<0.001$) or stereologically (WT: 0.8±0.3% versus KO: 3.3±0.2%; $P<0.001$, $n=6$ submandibular glands from three littermate mice in both groups). Furthermore, at E17.5, the percentage of apoptotic profiles was increased in GFRα2-KO mice (WT: 0.05±0.01% versus KO: 0.85±0.13%; $P=0.01$, $n=4$ submandibular glands analyzed from two mice in both groups) (Fig. 4).

Discussion

Our results show that GFRα2 signaling is important *in vivo* for the survival of parasympathetic intrapancreatic neurons during late fetal development and submandibular neurons around birth. Thus, increased apoptosis contributes to the loss of intrapancreatic and submandibular neurons observed in GFRα2-KO mice. Signaling via GFRα2, by contrast, does not seem to influence the amount of PCD in sympathetic paravertebral, sensory (DRG) or ENS ganglia during fetal development. In addition, we show that, despite their proposed common developmental origin, intrapancreatic but not enteric neurons undergo PCD during normal fetal development.

Activated caspase-3 and TUNEL are established markers used to study ontogenetic neuronal cell death (Srinivasan et al., 1998; Oppenheim et al., 2001; Urase et al., 2003). Of these, activated caspase-3 seemed more suitable than TUNEL to distinguish apoptotic neurons in the small peripheral ganglia because TUNEL-positive cells in the ganglia were often PGP9.5-negative, presumably because TUNEL detects cells at a later stage of death than activated caspase-3. Other pan-neuronal markers (such as Hu) could remain detectable during the later cell death (Lee et al., 2001). Although activated caspase-3 and TUNEL may not detect the full set of neuronal death (e.g. Borsello et al., 2002), to our knowledge TUNEL- and caspase-negative naturally occurring developmental death of neurons has not been clearly demonstrated (Yuan et al., 2003). For example, the normal death of embryonic chick ciliary ganglion neurons is morphologically non-apoptotic, classified as cytoplasmic or necrotic type of PCD (Pilar and Landmesser, 1976), but seems however detectable by TUNEL (Lee et al., 2001). Nevertheless, the possibility of non-apoptotic PCD of the developing enteric neurons cannot be fully excluded.

Future studies are warranted to address why intrapancreatic and enteric neurons differ with respect to ontogenetic caspase-3-mediated apoptosis and GFRα2 signaling even though the neuronal precursors in pancreas migrate from the fetal bowel. Enteric neurons are able to die via apoptosis, e.g. following injury (Boyer et al., 2005; Anitha et al., 2006), suggesting that the lack of ontogenetic PCD in enteric neurons may not

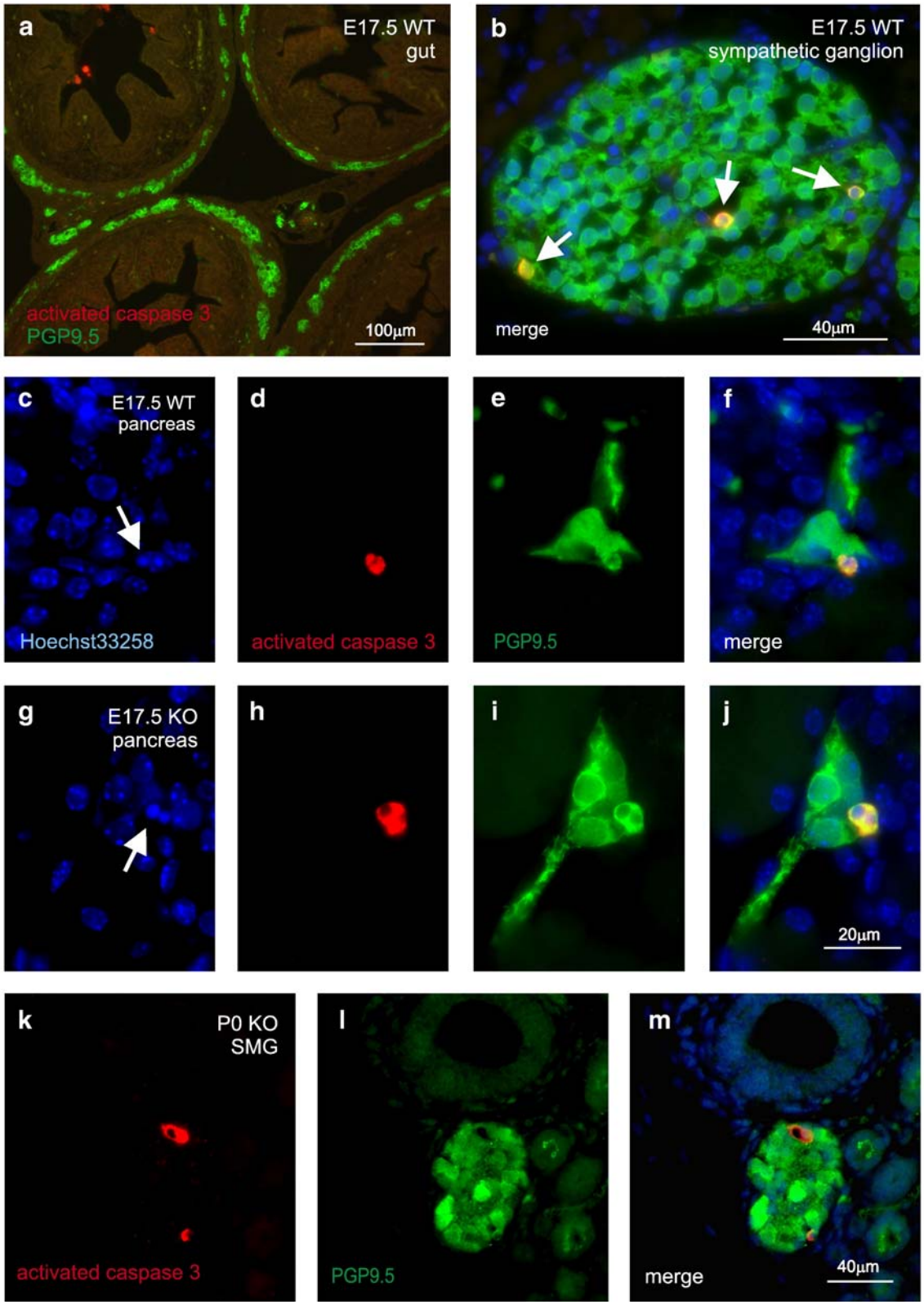

Fig. 5. Examples of mouse peripheral ganglia stained for activated caspase-3 (red) and PGP9.5 (green). DNA is stained with Hoechst 33258 (blue) to reveal the nuclear pyknosis of apoptotic cells. (a) Absence of apoptotic cells in the developing ENS. In this example of E17.5 wild-type myenteric plexus, none of the PGP9.5-positive myenteric neurons stains for activated caspase-3. (b) Apoptotic neurons in a paravertebral sympathetic ganglion of E17.5 wild-type fetus (arrows). (c–j) Apoptotic neurons with pyknotic nuclei (arrows) in intrapancreatic ganglia of both E17.5 wild-type (c–f) and GFRα2-KO (g–j) mice. (k–m) Apoptotic activated caspase-3-positive cells in newborn GFRα2-KO submandibular ganglion.

be explained by the lack of death machinery. The perpendicular migration of a subpopulation of enteric precursors to the pancreas (and to the submucosa) requires netrin/DCC signaling (Kirchgessner et al., 1992; Jiang et al., 2003). It is unclear whether the enteric precursors destined to become pancreatic neurons are predetermined (and different from their neighbors in the fetal bowel) or do they change their phenotype and become survival-dependent on GFRα2 signaling in response to signals they receive from the pancreatic microenvironment. Interestingly, similar to the intrapancreatic neurons, submucosal cholinergic neurons in the stomach require GFRα2 signaling for target innervation (our unpublished data). Thus it remains to be studied whether the submucosal subpopulation of enteric neurons might require GFRα2 signaling also for survival during postnatal development.

Neurturin mRNA is expressed in the developing mouse pancreas already at E14, but its expression is increased at E16 and E18 (Golden et al., 1999). Notably, the increase in neurturin expression coincides with the time of increased cell death in GFRα2-KO intrapancreatic ganglia (E16.5). Soon after (at E17.5), a significant decrease in the number of PGP9.5-positive cells is seen in the GFRα2-KO pancreas. Similarly, the increase of neurturin expression in the fetal submandibular salivary gland from E14 onwards (Golden et al., 1999) (and our unpublished data) precedes the increase in death of submandibular neurons in GFRα2-KO mice (this study) and is accompanied by a decrease in their number as compared with wild-type mice (Rossi et al., 2000). Thus, neurturin, the primary, and *in vivo* the only-known ligand signaling via GFRα2 (Heuckeroth et al., 1999; Rossi et al., 1999; Airaksinen and Saarma, 2002), is the most likely target-derived survival factor for the intrapancreatic and submandibular neurons during fetal development. However, as GFRα2-KO mice develop a postnatal growth retardation (Rossi et al., 1999) that is not observed in neurturin-KO mice (Heuckeroth et al., 1999), a ligand other than neurturin, possibly GDNF, might also activate Ret via GFRα2 in some tissue(s) important for growth.

We show that the majority of intrapancreatic neurons in GFRα2-KO mice are lost between E16 and birth. Is the increase in neuronal cell death sufficient alone, or do additional mechanisms, such as reduced migration or proliferation of neuronal precursors, contribute to the deficit? Our results suggest that the migration of enteric precursors from the bowel to the developing pancreas is normal in GFRα2-KO mice since no difference was present in the number of PGP9.5-positive intrapancreatic profiles between the genotypes at E15.5, when the number of intrapancreatic neuronal profiles is more than half of the maximum number counted at E17.5. This is consistent with the idea that most enteric crest-derived cells migrate into the pancreas from E13 to E15 (Jiang et al., 2003), and thus a possible defect in migration after E15.5 would not significantly contribute to the deficit. Moreover, our analysis of the mice after BrdU labeling indicated no difference between genotypes in the number of proliferating Phox2b-positive neuronal precursors in the pancreas at E15.5 or E17.5, making it unlikely that reduced precursor proliferation contributes to loss of intrapancreatic neurons. Assuming that apoptotic neurons are positive for activated caspase-3 (and PGP9.5) for only a few hours (during the late phase of death), we propose that the up to fivefold increase in apoptosis in GFRα2-KO intrapancreatic ganglia (as compared to wild-type) is sufficient to account for most, if not all, of the neuronal loss.

We also found an increased number of caspase-3-positive apoptotic neurons in the SMG of GFRα2-KO mice. Although the increased apoptosis could be secondary due to lack of target innervation (Rossi et al., 2000), a direct survival promoting role of GFRα2 would be consistent with previous reports that NRTN supports the survival of SMG neurons *in vitro* (Cacalano et al., 1998). Wild-type SMG had few apoptotic neurons at E17.5 (0.05%) and at birth (~0.5%), suggesting that PCD is not a major determinant of SMG neuron number during late fetal development, but it remains possible that additional SMG neurons undergo PCD postnatally.

In conclusion, we have demonstrated that neurons in two different parasympathetic ganglia depend on GFRα2 for survival. Thus, naturally occurring PCD in parasympathetic neurons is dependent on a limited supply of presumably neurturin signaling via GFRα2 for survival. Most parasympathetic neurons require GFRα2 signaling for target innervation and cell size, but some (e.g. in the sphenopalatine ganglion) neurons apparently do not need it for survival (Rossi et al., 2000). We hypothesize that a correlation exists between the extent of cell death occurring naturally and that occurring in GFRα2-KO mice in different parasympathetic ganglia. Another area for future research is uncovering the mechanism that prevents some parasympathetic and enteric neurons from undergoing PCD even if they depend on GFRα2 signaling for target innervation and cell size.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.02.016.

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